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The presence of antibodies to oxidative modified proteins in serum from polycystic ovary syndrome patients

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Summary

Polycystic ovary syndrome (PCOS) affects 5–10% of women of reproductive age. Free radicals, as a product of oxidative stress, impair cells and tissue properties related to human fertility. These free radicals, together with the oxidized molecules, may have a cytotoxic or deleterious effects on sperm and oocytes, on early embryo development or on the endometrium. Aldehyde-modified proteins are highly immunogenic and circulating autoantibodies to new epitopes, such as malondialdehyde (MDA), may affect the reproductive system. Autoantibodies or elevated reactive oxygen species (ROS) in serum are often associated with inflammatory response. The purpose of this work is to investigate whether PCOS women show increased levels of oxidized proteins (protein–MDA) and anti-endometrial antibodies (AEA) in their sera, compared with control patients, and to determine whether AEA specificity is related to oxidized protein derivatives. Sera from 31 women [10 patients with PCOS (PCOS group) and 21 women with male factor of infertility (control group)] were chosen from patients attending for infertility. Anti-endometrial antibodies were determined by enzyme-linked immunosorbent assay (ELISA) with an endometrial cell line (RL-95). Antibodies against MDA modified human serum albumin (HSA–MDA) were also determined by ELISA. Oxidized proteins (protein–MDA) in serum were determined by a colorimetric assay. Patients with PCOS have significantly higher levels of AEA and anti-HSA–MDA, as well as oxidized proteins (protein–MDA) in serum than control patients. For the first time, we describe an autoimmune response in PCOS patients, in terms of AEA. The evidence of protein–MDA in the serum of these patients, together with the increased antibody reactivity to MDA-modified proteins (HSA–MDA) *in vitro*, supports the conclusion that oxidative stress may be one of the important causes for abnormal endometrial environment with poor embryo receptivity in PCOS patients.

Keywords: anti-endometrial antibodies (AEA), autoimmunity, malondialdehyde (MDA), oxidative stress, polycystic ovary syndrome (PCOS)

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Introduction

Polycystic ovary syndrome (PCOS) affects 5–10% of women of reproductive age [1]. PCOS is a heterogeneous condition with several signs and symptoms and a controversial diagnosis that differs between countries [2], associated with hirsutism, amenorrhoea and obesity, and it is a common cause of infertility [1–5]. Diagnosis of PCOS is established according to the dysregulation of ovarian hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), as well as insulin resistance and hyperinsulinaemia. Laparoscopy, ovarian biopsia and histological features are the most important factors for clinical diagnosis. Morphological and histological studies show hyperplasia of internal theka, cortical stroma and tunica albuginea (TA), where many subcapsular follicles under TA are localized. Most primary infertile PCOS women have classic anovulation, obesity, high LH/FSH ratio and high testosterone levels. According to ultrasonography, these patients have 8–10 follicles with a diameter of approximately 10 mm.

Anovulation and infertility may be indicative of PCOS, but it needs to be confirmed by laparoscopy and biochemical analysis [1,6–8].

Insulin resistance is a common but not invariable finding in PCOS. However, insulin resistance may be a key factor in the pathophysiology of PCOS that exacerbates and underlies metabolic abnormality [9–11]. In many pathologies oxidative stress is considered to be one of the main causes of molecular damage to cellular and tissue structures. It is known to be increased in patients with diabetes [12,13] and in endometrial tissues from infertile patients with endometriosis [14–17]. The pathological conditions of diabetes with obesity have been associated widely with reactive oxygen species (ROS) and reactive carbonyl species (RCS) [13]. Free oxygen radicals play a role in several infertility-related diseases [18].

Patients with ovulatory dysfunction have a potentially autoimmune process associated with decreased fertility. PCOS is related to hormonal dysregulation as well as to autoimmune mechanisms. Reimand [19] investigated the prevalence of common organ-non-specific and organ-specific autoantibodies in women with reproductive failure due to the most common causes: polycystic ovary (PCO), PCOS and endometriosis, as well as in those patients with unexplained infertility. Tung [20] reported the production of oocyte autoantibodies in a murine model resulting in ovarian failure. We have published previously the presence of anti-endometrial antibodies (AEA) in the serum of infertile patients with ovulatory dysfunction, endometriosis or tubal obstruction [21].

Oxidative stress occurs when the production of ROS exceeds the endogenous anti-oxidant defence. ROS-induced peroxidation is the key of chemical and structural modifications of biomolecules, including circulating and structural proteins or lipids. During oxidative damage, reactive lipid peroxidation products can form adducts with free amino groups of lysine and other amino acids [22–25]. Aldehyde-modified proteins are highly immunogenic and circulating autoantibodies towards new epitopes, such as malondialdehyde (MDA), may be detected [25,26]. This process affects the reproductive system in various stages. Autoantibodies or elevated ROS in serum are often associated with an inflammatory response [16]. Evidence of oxidative stress and oxidatively modified lipid proteins is found in the peritoneal fluid of patients with endometriosis [16,27]. Autoantibodies to oxidatively modified proteins have also been detected in women with endometriosis [16,19,28]. The antigenicity is attributed to specific modified epitopes and not to the protein backbone [16].

Based on these considerations, we aimed to investigate whether PCOS women show increased levels of oxidized proteins (protein–MDA) and AEA in their sera compared with control patients. Our interest was to determine if AEA specificity was related to oxidized protein derivatives.

Materials and methods

This study included serum samples from 31 infertile women aged 32–46 years from the Department of Gynecology and Obstetrics at Charles University of Pilsen (Czech Republic). The study was approved by the institutional ethics committee, and informed consent was obtained from the women included in the study. Blood was taken at 12–14 days of the menstrual cycle. Clinical diagnosis of PCOS was based on the hormone profile, a history of slight menstrual irregularity, anovulation (according to the blood hormonal profile made on days 2, 14 and 26, basal vaginal temperature, functional vaginal cytology and ultrasonography), hirsutism and obesity in all women. Laparoscopy and ovarian biopsy were performed. All infertile patients in our study had no endometriosis. Body weight and height were also determined, and body mass index (BMI) was computed in all women as: weight (kg) × height (m^2).

Ovarian biopsy with histological analysis confirmed the polycystic ovary diagnosis in 10 patients. This PCOS group ($n = 10$) showed a high LH/FSH ratio and increased testosterone levels. Twenty-one women with no PCOS diagnosis and with male factor as a major cause of infertility comprised the control group ($n = 21$).

Determination of AEA

Adenocarcinoma endometrial cell line RL95-2 (CRL-1671 from the American Type Culture Collection (ATCC, Manassas, VA, USA) was used for AEA evaluation. Human epithelial cell line Hela (CCL-2 from ATCC), an epithelial non-endometrial cell line, was included as a control to detect unrelated antibodies to endometrial cells. Both cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with growth factors, penicillin (100 U/ml), streptomycin sulphate (100 μg/ml), amphotericin B (0·25 μg/ml) and 10% fetal calf serum [19]. Cells were cultured in 96-well flat-bottomed tissue culture-treated plates (Costar, Cambridge, MA, USA), 100 μl/well (5×10^4 cells/well) for 24 h at 37°C and 10% CO_2.

The enzyme-linked immunosorbent assay (ELISA) procedure has been described by Benet-Rubinat *et al.* [29]. For this assay, cells in culture plates were fixed with 100 μl/well of 0·25% glutaraldehyde solution. After a 30-min incubation at 4°C, the fixing solution was discarded and the wells were washed with 200 μl/well of phosphate-buffered saline (PBS) with 0·05% Tween-20 (PBS–Tween-20). Washings with PBS–Tween-20 were repeated three times at 5-min intervals after each step of the immunoassay. All incubations were carried out in a moist chamber.

Before adding the serum, a blocking solution was added to eliminate non-specific background reactions. Blocking solution consisted of 1% bovine serum albumin in PBS–Tween-20. The culture plate was incubated for 60 min at 37°C. The

wells were then washed with PBS–Tween-20 and 100 µl (1/100 in PBS dilution) of the serum was added in triplicate and incubated for 3 h at 37°C.

After incubation and washing of the culture plates, 100 µl of sheep anti-human immunoglobulin G (anti-human IgG) conjugated with horseradish peroxidase (The Binding Site, Birmingham, UK) at a 1/1000 dilution was added to each well and was incubated for 60 min al 37°C. The culture plates were washed and then chromogenic substrate (0·8 mM 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH, Sigma-Aldrich Inc., Ann Arbor, MI, USA), 40 mM 3-dimethylaminobenzoic acid (DMAB, Sigma-Aldrich), 3 mM H_2O_2 and 0·1 M phosphate buffer, pH 7·0) was added for 15 min at room temperature. The reaction was stopped with 50 µl/well of 2 N sulphuric acid (H_2SO_4, Sigma), and the absorbance (A) at 620 nm was determined automatically with a multi-scan plate reader (Anthos Labtech Instruments, Salzburg, Austria). Positive and negative sera were used in each ELISA plate determination. Autoantibody titres are expressed in absorbance units (A_{620}).

Detection of MDA–protein in serum

MDA was monitored by colorimetric assay. The evaluation of MDA was based on the Yagi method [30], which measures the MDA bound to protein. One hundred µl of each serum sample was precipitated with 800 µl N/12 sulphuric acid and 100 µl of 10% phosphotungstic acid (p/v). After 5 min at room temperature, the samples were centrifuged at 900 ***g*** for 10 min. Supernatant was removed and the total protein in the sediment was precipitated again in the same conditions. After centrifugation, the sedimented protein was diluted in 800 µl of distilled water. Then, 10 µl of 0·14 mM ethylenediamine tetraacetic acid (EDTA), 80 µl of 0·2% butylated hydroxytoluene (p/v) in ethanol (BHT) and 200 µl of 1% thiobarbituric acid (p/v) (TBA) were added and incubated in a boiling waterbath for 60 min. A sample of 600 µl was collected and added to 600 µl of butanol, and centrifuged again at 900 ***g*** for 15 min. Finally, 250 µl were removed from the butanolic phase and absorbances at 540 and 620 nm were determined with the Anthos ELISA reader (Cultek SL, Madrid, Spain); 1,1,3,3-tetraethoxypropane diluted in distilled water was used as standard. A standard curve (0·05, 0·1, 0·25, 0·50, 1 nM) was included in every assay, and correlation coefficients of $r = 0·992$ were obtained. All the samples were repeated three times and the final results shown are the mean values.

Oxidative modification of HSA–MDA

HSA to a final concentration of 5 mg/ml in 0·01 M of PBS containing 0·01% ethylenediamine tetracetic acid (pH 7·4) was incubated with 20 mM of MDA at 37°C [31]. The solution was dialysed at 4°C against 1000 volumes of PBS with four changes during 24 h to eliminate free MDA. Dialysis was removed and protein oxidation was determined by the MDA assay [30]. The total protein was determined by the method of Lowry *et al.* [32].

Determination of anti-HSA–MDA antibodies in serum

Anti-HSA–MDA antibodies in serum were determined by ELISA. Polystyrene microwell plates (Maxisorp, Nunc, Fisher Scientific, St Louis, MO, USA) were coated with 100 µl/well of HSA–MDA (20 µg/ml) in carbonate buffer, pH 9·6 at 4°C overnight. ELISA was performed in the same conditions described for AEA analysis. In parallel, unmodified HSA was used to evaluate background binding to the protein alone. The final data express the specific antibodies to oxidative MDA modifications in proteins.

Statistical analysis

Statistical analysis was performed with the SPSS package (version 11·9, SPSS Inc., Chicago, IL, USA). Data are expressed as mean values ± s.e.m. Statistical analysis for significant differences between two groups was obtained by Student's *t*-test. When multiple groups were compared, one- or two-way ANOVA was used, followed by a Student–Newman–Keuls test whenever applicable. In all instances, a value of $P < 0·05$ was considered statistically significant.

Considering the difficulties in obtaining samples from patients with PCOS, before the formal study was begun a preliminary study was performed with four patients and four controls. The AEA, MDA–proteins and anti-HSA–MDA were evaluated in their sera. According to the values obtained, the minimum number of patients necessary to demonstrate significant differences in these parameters between the two groups of study were calculated (PC-size program), accepting a value of $\alpha = 0·05$ and a β error = 0·10 (power analysis of 0·90). Analysis of the data showed that 10 patients per group would be adequate to demonstrate that AEA, MDA and anti-HSA–MDA values were higher in the serum of PCOS patients than in controls. Therefore, experiments were performed with the 10-patient PCOS group and 21 controls.

Results

No statistical differences were found in mean age (years) between the control group 31·05 ± 2·5 *versus* the PCOS group 28·6 ± 3·7 or in the BMI (kg/m^2) between the control group 24·55 ± 4·8 *versus* the PCOS group 22·05 ± 2·2.

Two epithelial cell lines were used for antibody evaluation: adenocarcinoma endometrial cell line RL95-2 for specific AEA determination and a human non-endometrial cell line, HeLa, as a control for antibody specificity. This allows discrimination between anti-endometrial and anti-epithelial antibodies. All autoantibodies recognized epitopes present in the endometrial cells, as detected by ELISA, but very low binding or no binding was found to epithelial cells (Fig. 1).

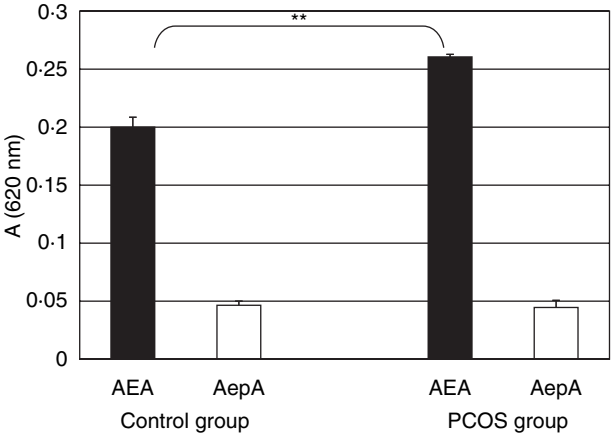

Fig. 1. Anti-endometrial (AEA) and anti-ephitelial (AepA) antibodies in the serum of patients. Results were expressed as absorbance units (A) at 620 nm. Symbols refer to mean values and bars to s.e.m. $^{**}P < 0{\cdot}01$.

We compared the levels of AEA in the serum from women in the PCOS group ($n = 10$) and patients with male factor (control group, $n = 21$). We observed that the mean value of AEA was significantly higher ($P < 0{\cdot}01$) in women with PCOS than in the control group. No statistical differences were found ($P < 0{\cdot}734$) when the mean value of anti-epithelial antibodies in both groups were compared (Fig. 1).

Levels of MDA bound to proteins in serum, as indicative of oxidative protein status, were compared in both groups. The mean value of protein–MDA was significantly higher ($P < 0{\cdot}001$) in the serum of patients with PCOS than in the control group (Fig. 2).

We detected significantly higher values ($P < 0{\cdot}05$) of anti-HSA–MDA in serum in the PCOS group ($0{\cdot}09 \pm 0{\cdot}03$) than in the control group ($0{\cdot}041 \pm 0{\cdot}03$), but we did not find any difference among the values for anti-HSA in both groups (Fig. 3).

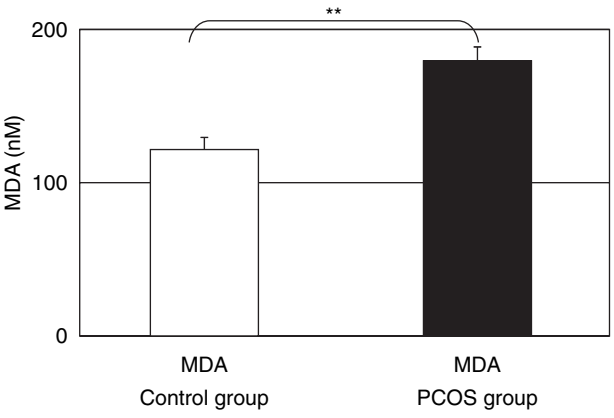

Fig. 2. Malondialdehyde (MDA) levels bound to proteins (protein–MDA) in the serum of patients. Symbols refer to mean values and bars to s.e.m. $^{**}P < 0{\cdot}01$.

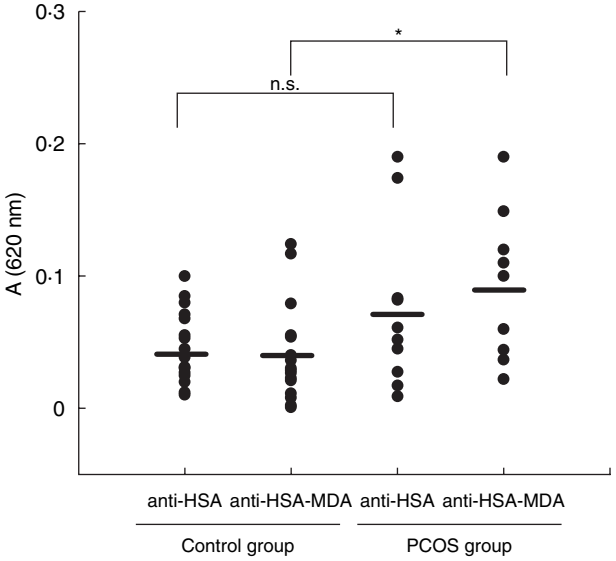

Fig. 3. Anti-human serum albumin (HSA) and anti-HSA-malondialdehyde (MDA) antibodies in serum of polycystic ovary syndrome patients. Results were expressed as absorbance units (A) at 620 nm; n.s. = not significant. $^{*}P < 0{\cdot}05$.

Discussion

Oxidative stress, with consequent production of high amounts of free radicals, may play an important role in the aetiology of infertility such as endometriosis, PCOS and miscarriages. Redox imbalance causes changes in biological molecules. The oxidative process produces chemical modifications (aldehydes, amino acid derivatives) in lipids and proteins that are part of cell membrane and other cellular structures. The consequence may be molecular damage to cell and tissue structures and the formation of a non-self structure or 'new epitope' in biological molecules [13,33–37].

Redox has been studied poorly in the reproductive system. ROS exert deleterious effects on sperm [38], sperm oocytes binding [39] and early stages of fertilized oocyte [40]. Szczepanska *et al.* [17] suggested that ROS plays a role in the pathogenesis of infertility in women with endometriosis. Her group argues that chemical modifications in proteins lead to the introduction of carbonyl groups. Protein modification by ROS is involved in the cause and progression of a number of physiological and pathological disorders [5,17]. As a consequence of an oxidative process, membrane lipid peroxidation appears and several metabolites, such as MDA, also appear. MDA is able to modify oxidatively proteins rich in Lys, Cys and His amino acids [41]. For this reason, we tried HSA protein rich in these amino acids to detect specific antibodies to oxidative protein modifications.

Our ELISA with HSA–MDA antigen showed that anti-HSA–MDA antibodies are present in patients with PCOS. Similar results were obtained by other authors in arteriosclerosis, diabetes, essential hypertension and renal failure

[42–44], but the clinical significance of these antibodies is hampered by their frequent presence, even in healthy controls [45].

Shanti *et al.* [16] hypothesize that autoantibody titres in serum to ox-LDL and other modified antigens are increased in women with endometriosis. Oxidatively modified antigens present in the serum are examples of the terminal degradation of protein 'damage' by peroxidized lipids. Shanti's group suggests that these autoantibodies are specific for the oxidized epitopes and not for the proteins that carry the epitopes. The autoantibodies recognize oxidatively modified epitopes on other proteins [16].

Our group found an AEA increase in the serum of patients with endometriosis, tubal obstruction and ovulatory dysfunction [19], and this AEA increase correlates positively with the degree of endometriosis in the endometriotic patients [28]. Higher binding of AEA to previously stressed endometrial cells is detected by ELISA, compared to the binding to non-stressed cells (data not published). In the present study, we found a significant difference in anti-HSA–MDA and AEA antibodies when comparing control and PCOS groups. We suggest that some autoantibodies to endometrial cells could be directed against MDA or other oxidized radicals in proteins or lipids of the endometrial membrane. These results are in agreement with those of Shanti *et al.* [16], who showed increased levels of autoantibodies to lipid oxidative modifications in women with endometriosis.

The anti-oxidant system has many components [18] that may be important in the study of infertility diagnosis. Anti-oxidants (uric acid, vitamins C, E and β carotene, α tocopherol, albumin) present in plasma contribute to redox equilibrium. The anti-oxidant status of plasma is the result both of the anti-oxidant levels and degree of oxidative stress [5]. Our data suggest that there is an oxidative status in PCOS. This is supported by high levels of MDA-modified proteins in PCOS serum and it could be related to the presence of anti-MDA protein antibodies in the serum of these patients. Therefore, some of the AEA reactivity could be to these oxidative modifications. Investigations comparing oocyte and embryo quality in women with PCOS report that, even though the quality of the embryos recovered was good, the fertilization rate was very poor.

Obesity is characteristic, and has been reported to occur in about half of PCOS patients. However, in our PCOS group we did not find elevated values of BMI if we compared them with the accepted reference values characteristic of this pathology. One possible cause of these low values found by our group could be attributed to the facts that these patients are younger than 30 years old and that the mean BMI is lower in European than in North American populations. The rate of obesity associated with PCOS has not been corroborated, but there is a growing impression that the incidence may be greater in the United States, as described previously by Chang [6].

PCOS patients seeking to become pregnant will have difficulties because of anovulation [2,46]. However, in the case of these patients having the opportunity to obtain a fertilized oocyte, they do not reach successful implantation. We describe, for the first time, an autoimmune response in PCOS patients in terms of AEA in serum. The presence of an increase of MDA bound to proteins in the serum of these patients, together with the increased antibody reactivity to MDA-modified proteins (HSA–MDA) *in vitro*, supports the conclusion that oxidative stress could play a role in poor embryo implantation of PCOS patients. The AEA generated in PCOS could recognize new protein oxidized epitopes generated during oxidative stress in the endometrial cells.

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